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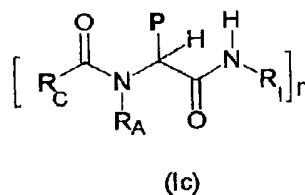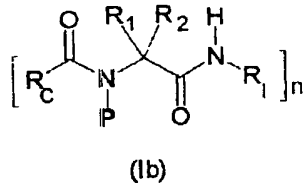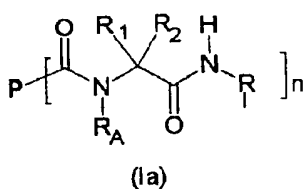ZIEGLER, THOMAS (DE).

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(54) NOUVEAUX CONJUGUES DE PROTEINES ET PROCEDE POUR LEUR PREPARATION
(54) NOVEL PROTEIN CONJUGATES AND PROCESS FOR THE PREPARATION THEREOF

(57)
The invention relates to novel protein conjugates of the formulae (1a-c). The invention further relates to a method for preparing the protein conjugates by reacting proteins with three low molecular weight components in a single reaction step, up to four reporter groups or ligands being introduced simultaneously at a defined stoichiometry and molecular distance and to applications of the novel protein conjugates.

(54) NOUVEAUX CONJUGUES DE PROTEINES ET PROCEDE POUR LEUR PREPARATION
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(Ia)   (Ib)   (Ic)

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Novel protein conjugates and process for the preparation thereof

5   Abstract

The invention relates to novel protein conjugates of the formulae (la-c). The invention further relates to a method for preparing the protein conjugates by reacting proteins with three low molecular weight components in a single reaction step, up to
10   four reporter groups or ligands being introduced simultaneously at a defined stoichiometry and molecular distance and to applications of the novel protein conjugates.

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Novel protein conjugates and process for the preparation thereof

Description

The invention relates to novel protein conjugates and a process for the preparation thereof. The novel protein conjugates comprise on the one hand a naturally occurring, synthetically modified or recombinant protein, enzyme, immunoglobulin, antibody, receptor protein or lectin and on the other hand simultaneously three further low molecular weight compounds selected from the group consisting of amines, carboxylic acids, isonitriles, aldehydes or ketones. The invention further relates to a process for preparing the novel protein conjugates by reacting a protein with the three low molecular weight components in a single reaction step, up to four reporter groups or ligands being introduced simultaneously in a defined stoichiometry and molecular distance and to applications of the protein conjugates obtainable in this way.

Background:

Linkage products of proteins and low molecular weight compounds, so-called protein conjugates, are applied in many different ways within the life sciences. In medicine they include, for example, protein conjugates which can be employed therapeutically and which consist of, for example, an antibody and a cytostatic. In diagnostics and biochemistry protein conjugates are employed in a great variety of fields for recognizing, labeling and quantifying the proteins themselves or their molecular receptors, messenger molecules and antagonists. In medical immunology protein conjugates, in particular of carbohydrate and nucleic acid antigens, are used for immunization (vaccination). The protein may serve on the one hand as a vehicle for presenting antigens and for the targeted transport of one or more low molecular weight compounds; on the other hand it is possible to employ the low molecular weight part of protein conjugates to specifically modify properties of the proteins themselves. The latter variant comprises, for example, specific modification of the physical properties of the protein, for example thermal stabilization or solubility characteristics of a catalytically active enzyme, catalytically active antibody (abzyme), therapeutically active protein or antibody or specific modification of the actual catalytic activity, specificity or general properties for recognizing a substrate, receptor or ligand. Thus the function of the protein part of the protein conjugates may

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extend from a mere vehicle (prodrug) via targeted transport (drug targeting) or ligand presentation (vaccines) to the catalytic activity or actual molecular recognition. Conversely, the function of the "low molecular weight" part of the protein conjugates extends from physical modification or stabilization via labeling using one or more characteristic and analytically quantifiable reporter groups (diagnostics) to the actual principle of action (e.g. in prodrugs) or multivalent ligands (e.g. in vaccines).

Most physiologically active proteins are already in the form of protein conjugates, in particular as conjugates with carbohydrates (glycoproteins), which underlines the biological importance of carbohydrates as binding partners of proteins. They are primarily bound on the protein surface by serine, threonine and asparagine and take part, for example, in cell recognition. The artificial attachment of low molecular weight structures, in particular also further carbohydrates, to so-called neoglycoproteins may be employed, for example, to specifically modulate cell recognition and to study the biological phenomena it is based on.

The available repertoire of methods for linking carbohydrates, active ingredients, reporter molecules, dyes, antigens, ligands etc. to proteins is based essentially on the presence of reactive amino, carboxylate, thiol or aldehyde groups (after oxidation) in proteins. Thus it is known already that proteins carrying free amino, carboxylate. thiol or aldehyde groups can be linked to low molecular weight compounds which themselves carry free carboxylate or amino groups to give protein conjugates (Advances in Carbohydrate Chemistry and Biochemistry 37, 225-281, 1980). Free protein amino groups are in particular the N termini of the proteins and the ε-amino groups of lysines which can be linked to low molecular weight carboxylic acids at least partially by amidation. Free protein carboxylate groups are in particular the C termini of the proteins and the carboxylate groups of the amino acids aspartic acid and glutamic acid, which are linked to low molecular weight amines at least partially by amidation. Cysteines present free thiol groups; free aldehyde groups of proteins are in particular oxidized carbohydrate components of proteins which can be linked to amines by reductive amination.

Numerous reagents are available as condensing agents for these reactions, in particular those agents which may be employed quite generally for amidation reactions in organic synthesis (Novabiochem catalog, 1999, page 264 ff.) such as, for example, carbodiimides, in particular EDC (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide). Additionally, bifunctional coupling reagents having two equivalent or two orthogonal active groups are used either as crosslinkers or for conjugation with

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any additional component (low molecular weight compound, additional protein, solid phase). Suitable active groups thereof are maleimides, imido esters, pyridyl disulfides, α-halo carbonyl compounds and aryl azides (Pierce catalog, 1997, pages 133-154).

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Enzyme labeling is needed, for example, in biochemistry for detection in immunoassays (ELISAs), in immunohistochemistry, for Western blotting and for DNA or RNA hybridization assays. A variety of biochemical, analytical and technical applications use in particular peroxidase (immunoassays, immunoblots, immunohistochemistry), alkaline phosphatase (immunoassays, immunoblots), β-galactosidase (immunoassays, immunoblots, immunocytochemistry of blood samples) and glucose oxidase (immunohistochemistry, biosensors).

A typical example of the known procedure is the preparation of conjugates of alkaline phosphatase (AP) by reacting in an aqueous buffer solution the reagent succinimidyl 4-[N-(maleimidomethyl)-cyclohexane]-1-carboxylate (SMCC) with free AP amino groups to give activated AP. It is then possible in a second step for a low molecular weight compound or a protein, for example an antibody, to be linked to the maleimide of the activated AP by free thiol groups. In another example, horseradish peroxidase (HRP) can bind to ligands or antibodies quite analogously.

Further known methods for preparing protein conjugates are reductive amination, for example by complex borohydrides or the reaction with reactive cumulative multiple bond systems. The latter include, for example, isocyanates. Thus so-called neoglycoproteins are prepared, for example, from BSA (bovine serum albumin) by reacting the protein with up to 200 equivalents of an isocyanate-modified sialyl-Lewis-X tetrasaccharide, yielding an epitope density of up to 16 (J. K. Welply, Glycobiology 4, 259-265, 1994). US 5,059,654 describes further known methods for linking proteins, in particular to solid phases such as polysaccharides. Typical processes for covalently linking polysaccharides to carrier proteins with the aim of vaccine production are based on analogous processes, in particular by employing bifunctional linkers (WO 99/18121). The following reference extensively describes common modification processes for glycoproteins and proteoglycans: B.Kuberan et al., Glycoconjugate J., 16, 271-281 (1999).

The mentioned known methods for preparing protein conjugates most often use very reactive electrophilic reagents (for example isocyanates, carbodiimides) reacting with the native nucleophilic groups of the protein (amine, carboxylate, thiol). In order to

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attach a nucleophilic antigen to a protein, for example for producing synthetic vaccines, a suitable bifunctional reagent more or less reverses its reactivity by amidating the nucleophile obtained after reductive amination of the oligosaccharide antigen, for example, using a succinimide active ester which for its part is linked via a

5   linker to an electrophilic maleimide, which reacts subsequently with the nucleophilic thiol groups of the protein. Using the known coupling reagents it is in principle possible also to attach a plurality of reporter groups or ligands to the protein, provided that the latter react as a mixture or successively in multiple process steps. It would not be possible, however, to control the stoichiometry of the protein

10   conjugates to be prepared in this way because not all of the protein's functional groups have exactly the same reactivity. It would also be impossible to control in this way the molecular proximity of ligands or reporter groups to be introduced in the protein.

15   On the other hand novel protein conjugates containing up to four further functional groups (e.g. ligands, antigens) or analytically detectable groups (reporter groups, e.g. dyes) would be desirable. All of these groups ought to be linked to the protein simultaneously in a single simple process step providing an exactly defined stoichiometry of the formed protein conjugate and a structurally defined molecular

20   distance of the functional or analytical groups.

The novel proteins Ia-c may also be in the form of mixtures with the aim of generating from an initial protein mixture, which was obtained from, for example, a cell or body fluid and fractionated by means of, for example, high-performance liquid

25   chromatography (HPLC) or two-dimensional gel or capillary electrophoresis, a specific detectable protein pattern significantly different from the pattern of the existing protein mixture owing to the performed modification.
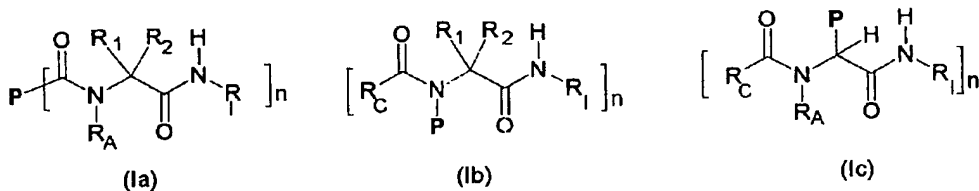
The abovementioned methods for preparing protein conjugates using bifunctional

30   reagents cannot fulfill these technical demands. The same is true for specific types of reagents of higher functionality: thus, for example, sulfo-SBED (Pierce, 1997, page 151) is a trifunctional molecule for preparing protein conjugates combining in one reagent an amino specific succinimide ester, a biotin ligand and an unspecific photoreactive group (phenylazido).

35   Furthermore, Endeavour *18*, 115 - 122 (1994) has already disclosed a reaction of low molecular weight amines with carboxylic acids, isonitriles and a carbonyl compound to give low molecular weight peptides (Ugi reaction). For this, soluble proteins have not been used as amino, carboxylic acid or aldehyde components up

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to now. In particular, isonitriles have not been used as condensing agents in combination with carbonyl compounds for this reaction up to now.

Description:

The invention relates to novel protein conjugates of the structures given in the formulae (la-c)

(la)   (lb)   (lc)

wherein **P** is a protein carrying free amino, carboxyl or aldehyde groups such as an albumin, immunoglobulin, antibody, avidin, streptavidin, hemocyanin, lectin, enzyme or serum glycoprotein,

and R_A is a radical derived from amines R_A-NH_2, where RA is an amino group-carrying linear or branched alkyl or cycloalkyl radical of up to 18 carbon atoms, an amino group-carrying alkaloid, peptide or protein, carbohydrate, nucleotide, nucleoside, steroid, terpene, porphyrin, chlorin, corrin, eicosanoid, pheromone, vitamin, in particular an n-(biotinamido)-n-alkyl radical where n = 2-8, an antibiotic, cytostatic, a dye molecule or a cryptand, in particular tris(bipyridinium)-europium(III).

and R_C is a radical derived from carboxylic acids R_C-CO_2H, where R_C is a carboxyl group-carrying linear or branched alkyl or cycloalkyl radical of up to 18 carbon atoms, carboxyl group-carrying alkaloid, peptide or protein, carbohydrate, nucleotide, nucleoside, steroid, terpene, porphyrin, chlorin, corrin, eicosanoid, pheromone, vitamin, in particular biotin, an antibiotic, cytostatic, dye molecule or a cryptand, in particular tris(bipyridinium)-europium(III).

and R_l is a radical derived from isonitriles R_l-NC, where R_l is an isonitrile group-carrying linear or branched alkyl or cycloalkyl radical of up to 18 carbon atoms, methoxycarbonylethyl, t-butoxycarbonylmethyl, phenyl, o-alkylphenyl, m-alkylphenyl, p-alkylphenyl, o-halophenyl, m-halophenyl, p-halophenyl, 2,3-dihalophenyl, 2,4-dihalophenyl, 3,4-dihalophenyl, o-alkoxyphenyl, m-alkoxyphenyl, p-alkoxyphenyl, o-arylphenyl, m-arylphenyl, p-arylphenyl, o-aryloxyphenyl, m-aryloxyphenyl, p-aryloxyphenyl, o-nitrophenyl, m-nitrophenyl, p-nitrophenyl, 1-naphthyl, 2-naphthyl,

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benzenesulfonylmethyl, p-toluenesulfonylmethyl, pyranosyl radical, furanosyl radical, nucleosyl radical, n-(biotinamido)-n-alkyl radical where n = 2-8 or a dye molecule,

and R_1 and R_2 are a radical R_1-C(=O)-R_2 derived from carbonyl compounds and can be simultaneously or independently of one another hydrogen, methyl, ethyl, propyl, i-propyl, cyclopropyl, butyl, i-butyl, t-butyl, cyclobutyl, pentyl, cyclopentyl, hexyl, cyclohexyl, 2-methylbutyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, methoxycarbonylethyl, t-butoxycarbonylmethyl, phenyl, o-alkylphenyl, m-alkylphenyl, p-alkylphenyl, o-halophenyl, m-halophenyl, p-halophenyl, 2,3-dihalophenyl, 2,4-dihalophenyl, 3,4-dihalophenyl, o-alkoxyphenyl, m-alkoxyphenyl, p-alkoxyphenyl, o-arylphenyl, m-arylphenyl, p-arylphenyl, o-aryloxyphenyl, m-aryloxyphenyl, p-aryloxyphenyl, o-nitrophenyl, m-nitrophenyl, p-nitrophenyl, 1-naphthyl, 2-naphthyl, oxiranyl, vinyl, propenyl, propen-2-yl, 2-penten-2-yl, 3-hepten-3-yl, penta-1,3-dienyl, phenylmethyl, 1-phenylethyl, 2-phenylethyl, cyclohexen-3-yl, cyclohexen-4-yl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyrrolyl, 3-pyrrolyl, halomethyl, dihalomethyl, trihalomethyl, 2-pyridyl, 3-pyridyl, 4-pyridyl,

and n = 1-15, preferably 1-10.

The invention further relates to a process for preparing the novel protein conjugates of the formulae (Ia-c) by reacting a protein (P) which is soluble or immobilized on a solid phase and which carries free amino, carboxylic acid or aldehyde groups with an amine R_A-NH_2, or carboxylic acid R_C-CO_2H, isonitrile R_I-NC and carbonyl compound R_1-C(=O)-R_2, where R_A, R_C, R_I, R_1 and R_2 are as stated above, in aqueous solution to give the corresponding protein conjugates (Ia), (Ib) or (Ic).

Here the respective structure variations (Ia-c) – as can be seen from the structure formulae – are obtained from the corresponding components as follows:

(Ia): from the protein (P), amine R_A-NH_2, isonitrile R_I-NC and carbonyl compound R_1-C(=O)-R_2,

(Ib): from the protein (P), carboxylic acid R_C-CO_2H, isonitrile R_I-NC and carbonyl compound R_1-C(=O)-R_2,

(Ic): from the oxidized protein (P), carboxylic acid R_C-CO_2H, amine R_A-NH_2 and isonitrile R_I-NC.

The process according to the invention is particularly suitable for reacting proteins (P) carrying free amino groups or free carboxylate groups or free aldehyde groups. Particularly suitable are the following proteins which are either free or bound to a solid phase:

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- carrier proteins to be linked to haptens (peptides or carbohydrates) as immunogenic epitopes, for example KLH (keyhole limpet hemocyanin), CRM (nontoxic diphtheria toxin variants) or albumins such as bovine serum albumin (BSA).

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- native or recombinant proteins of microbial origin used in immunology, for example proteins A, G or A/G, which recognize the Fc part of immunoglobulins, for example in IgG. Furthermore, avidin and streptavidin and lectins such as, for example, concanavalin A or wheat-germ agglutinin (WGA) and peptidoglycans.

- immunoglobulins IgG, IgM, IgA, also in oxidized form, for example oxidized IgG.

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- enzymes such as, for example, pepsin, papain, trypsin, ficin, chymotrypsin, lipases, esterases, oxidoreductases, transaminases, glycosidases, e.g. β-galactosidase, glycosyltransferases, amidases, hydantoinases, horseradish peroxidase (HRP), alkaline phosphatase (AP).

20 In the process according to the invention the protein (P) is reacted with the amine R_A-NH_2 or the carboxylic acid R_C-CO_2H, and the isonitrile R_I-NC and the carbonyl compound R_1-$C(=O)$-R_2 in such a way that the amine R_A-NH_2 or the carboxylic acid R_C-CO_2H and the isonitrile R_I-NC are added successively to a solution or suspension of (P) in aqueous buffer containing the other components in at least
25 molar amounts to the protein in each case. Here each of the required components amine R_A-NH_2, carboxylic acid R_C-CO_2H, isonitrile R_I-NC and carbonyl compound R_1-$C(=O)$-R_2 are employed in a 10-10,000 fold molar excess over protein (P), preferably in a molar ratio of 100-1,000, particularly preferably in a ratio of 200-1000.

30 Suitable reaction solutions are aqueous buffers such as, for example, 0.001 – 1.0 molar solutions of sodium or potassium dihydrogen phosphate and disodium or dipotassium hydrogen phosphate or solutions of tris(hydroxymethyl)aminomethane and hydrochloric acid, and particularly suitable are buffer solutions for the pH range between 5 and 9, particularly preferably between pH 6 and pH 8.

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In the process according to the invention methanol, ethanol, propanol, i-propanol, butanol, ethyl acetate, methyl acetate, dimethylformamide, acetonitrile, dimethylsulfoxide or sulfolane may be added to the buffer as cosolvents in quantities

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of 0.1-20% by volume, depending on the solubility requirements of the reactants. The reaction temperature is between 0°C and 90°C, preferably 10°C to 40°C, and is particularly preferably room temperature.

5   The crude protein conjugates (Ia-c) obtainable from the process according to the invention may be further purified by dialysis using aqueous buffer solutions or pure water and biochemical chromatography processes familiar to the skilled worker and then put to further use.

10   To detect the exact structure of the products (Ia-c), that is to analytically determine the mean number (n) of the components bound to the protein (P), the molecular weight is directly measured by means of MALDI-TOF mass spectrometry or one or more components bound to the protein are selectively determined. In this way it is possible, for example to measure the amount of a dye molecule introduced by the

15   components R_A-NH_2, R_C-CO_2H or R_I-NC in a simple manner through the UV extinction. An analogously introduced nucleotide may be quantified according to the familiar biochemical methods, where appropriate after amplification by means of PCR methods.

20   The resulting calculated number (n) describes the stoichiometry, i.e. the so-called mean epitope density on the protein (P) of formulae (Ia-c).

Thus the process according to the invention can be carried out in practice, for example, such that initially a protein (P) such as, for example, bovine serum albumin

25   is dissolved at room temperature (appr. 20°C) in 0.1 molar phosphate buffer (pH 7.5) containing the carbonyl compound R_1-C(=O)-R_2 in a molar ratio of 100 - 4000. Subsequently, R_A-NH_2 or R_C-CO_2H and R_I-NC are added successively to this solution in the appropriate molar ratio of 100 - 4000 (relative to P). After a reaction time of two days at room temperature, excess low molecular weight reaction

30   components are removed by dialysis using buffer or pure water or by chromatography. The solution or suspension of the novel protein conjugate obtainable in this way is then analytically characterized as described above.

Variation of the total of 4 reaction components 1. amine R_A-NH_2, 2. carbonyl

35   compound R_1-C(=O)-R_2, 3. carboxylic acid R_C-CO_2H and 4. isonitrile R_I-NC, the protein (P) being employed either as an amine R_A-NH_2, carbonyl compound R_1-C(=O)-R_2 or as carboxylic acid R_C-CO_2H depending on the selection of the remaining 3 reaction components employed in excess, leads to manifold possibilities

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of attaching almost any components to (**P**), up to four different types of ligands (e.g. biotin) and/or reporter groups (e.g. dyes) being introduced at an exactly defined stoichiometric ratio of 1:1:1:1 and a defined molecular distance relative to each other and relative to the protein by varying the respective radicals <u>R.</u> It is possible here to adjust the molecular ratios further by selecting the spacer lengths in the radicals R_A, R_1, R_2, R_C and R_I.

The following formulae illustrate a small selection of the various possibilities resulting therefrom, for example for conjugating 2 different dyes (F1. and F2) and any further ligand, for example, biotin (B):

(Ia)　　　　　　　(Ib)　　　　　　　(Ic)
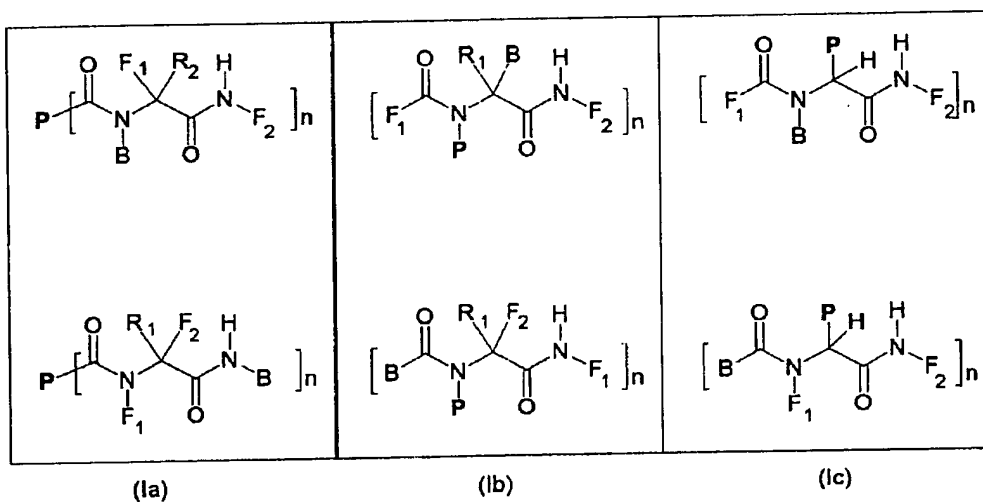
One example is, for example, to use a dye label as carboxyl component R_C-CO_2H and a biotin ligand as amine component R_A-NH_2:
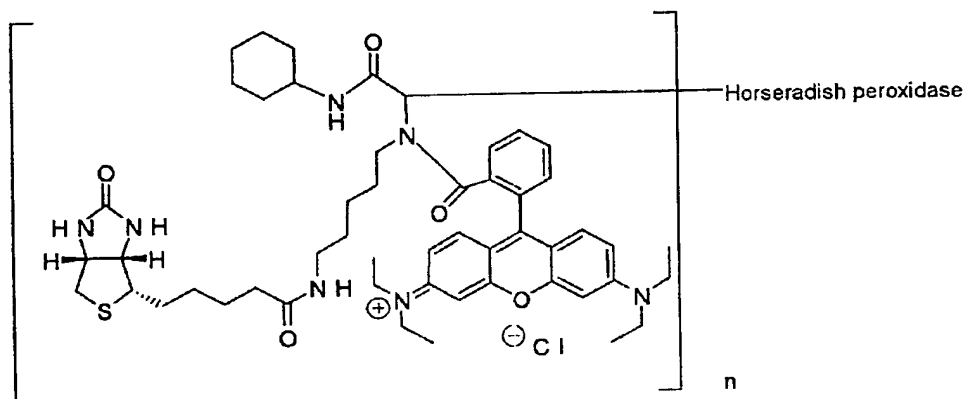
This example illustrates the application potential of the protein conjugates according to the invention and the simplicity of the process of their preparation: the four reaction components

1. Oxidized protein horseradish peroxidase as carbonyl component $HC(=O)-R_2$,
2. Rhodamine dye label as carboxyl component R_C-CO_2H
3. Biotin ligand as amine component R_A-NH_2
4. Cyclohexyl isonitrile as R_I-NC

are combined simply in the desired stoichiometric ratio and reacted in aqueous buffer under mild conditions. The outlined course of reaction and the structure of the novel protein conjugate can now be characterized exactly using independent functional determination methods for the two reporter groups (biotin, dye): thus, molar mass determination by means of MALDI-TOF gives a mean epitope density of $n = 3.4$ which is confirmed by a biotin ELISA assay ($n = 3.3$) and UV extinction measurement ($n = 3.5$). The enzyme activity (residual activity 93%) is to a large extent retained in the enzyme assay.

Conversely, it would also be possible to employ rhodamine B as amine component R_A-NH_2 via a suitable linker and biotin as carboxyl component R_C-CO_2H, to mention only one of the various possible variations.

Thus it is possible for the stoichiometrically present reporter group to be employed for direct internal determination of another reporter group and vice versa, thereby improving the accuracy of the determinations. Thus, the dye in the abovementioned example may serve to determine the degree of biotinylation directly and internally and vice versa. This may be much simpler than the familiar methods for determining biotin in proteins which employ indirect methods: thus, for example, the HABA-avidin method (HABA = 2-hydroxyazobenzene-4'-carboxylic acid) uses binding to avidin to form a colored complex (absorption at 500 nm) which is displaced by free biotin. A plurality of measurement points form a standard curve from which the degree of biotinylation is determined.

Thus it is also possible for example to use the protein conjugates (Ia-c) according to the invention in various ways in the biotin-avidin complex (ABC) system for detecting and quantifying antigens, for example on solid phases. This naturally also includes all biochemical assay methods based on this system such as, for example, the classical ELISA assay, fluorescence-activated cell sorting (FACS), affinity chromatography and Western blotting.

Proteins and enzymes frequently used in these systems are avidin, streptavidin, horseradish peroxidase, glucose oxidase and alkaline phosphatase. Frequently used

dyes are, for example fluorescein. (FITC), rhodamine B and Texas red, but variants of these molecules having improved fluorescence properties may also be used in accordance with the process according to the invention, for example sulforhodamine conjugates (US 5,846,737).

It is therefore possible for the protein conjugates (Ia-c) according to the invention to be integrated in various ways into the immunological methods used in clinical diagnostics, for example, for monitoring plasma levels of analytes in the blood, checking hormonal control systems (thyroid function, fertility), detecting viral infections (hepatitis, HIV) or tumor-associated proteins (tumor aftercare). Macromolecules such as peptides, proteins and nucleic acids are determined in general by heterogeneous immunoassays (e.g. solid phase carrying an antibody), while for low molecular weight substances homogeneous methods such as fluorescence polarization assay (FPIA) or enzyme multiplied immunoassay (EMIT) are used as well. Newer methods have an increased sensitivity by simultaneous binding of the analyte (e.g. a tumor marker protein) to a monoclonal antibody conjugated with a europium(III) complex and to a further antibody coupled to a dye molecule (allophycocyanin). According to the *Förster* theory, the absorbed energy of monochromatic light, due to the molecular spatial proximity of lanthanide complex and dye molecule, leads to a radiation-free energy transfer from the former to the latter during binding and thus causes a long-lived and amplified emission (time-resolved amplified cryptate emission assay, TRACE) (Bioforum, *1-2*, 1998, 14-16.). Similarly, the spatial proximity of one or more dyes (multiple chromophoric donor groups) to a fluorescence acceptor dye may be used for signal amplification (US 5,849,489). A particularly sensitive trace analysis of biomolecules is based on the so-called fluorescence cross correlation (FCS) spectroscopy comprising the light amplification by two fluorogenic dyes in defined spatial proximity. Excitation by two corresponding laser sources leads to energy transfers and amplified fluorescence emission of the labeled molecule, which is measured by photon detectors in a confocal arrangement. Examples of dyes used are rhodamine green and Cy5 (both available from Amersham Life Sciences). R. Rigler et al., J. Biotechnol., 63, 97-109 (1998) describe the fundamentals of this method which was used to quantify a few copies of an amplified DNA.

Since the process according to the invention allows linking of up to 4 reporter groups in defined stoichiometry and adjustable molecular distance, it is possible to expect from corresponding protein conjugates (Ia-c) having these types of dyes (examples of corresponding derivatives in US 5,849,489) a greatly increased sensitivity in the

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detection of the protein itself or of a bound ligand or of an analyte to be determined in an immunoassay.

The process according to the invention is suitable in particular also for specific
5   conjugation of saccharides having a defined epitope density on the protein (see Examples). The latter may be determined, for example, in one of the reaction components by the stoichiometric simultaneous attaching of a dye reporter molecule. This opens up the possibility of attacking in a controlled manner to, for example, suitable proteins such as CRM (nontoxic diphtheria toxin variant) or the KLH protein
10   a plurality of saccharide antigens of, for example, various bacterial or viral serotypes (e.g. influenza, hepatitis) to give vaccines of even higher valency or a plurality of tumor associated antigens for tumor vaccines.

The process according to the invention is further suited to covalently linking one or
15   up to three different cytostatics to a protein, for example to a tumor-specific antibody or to an enzyme, for example to a glycosidase, or to a glycoprotein recognized by a cell-specific lectin such as, for example, asialoglycoproteins recognized by liver cell lectins.

20   The process according to the invention is further suited to reacting protein mixtures with the aim of creating a specifically detectable protein pattern after appropriate fractionation of this protein mixture in order to analytically characterize the proteome of an organism (proteomics). In this case, the process step according to the invention is simply added to the sample preparation before developing the usual two-
25   dimensional electrophoresis chromatogram (so-called 2-DE map). The usual methods for visualizing the protein spots on gels as stationary phases employ Coomassie blue or silver nitrate (staining) and fluorescent dyes. The proteins are fractionated in two dimensions according to their molar masses and isoelectric points (pI values), basic proteins being detected, for example, up to pI 9 and acidic proteins
30   up to pI 4-5.

Proteins having a pI beyond these values are separated or detected less well or not at all. Here the process according to the invention offers a technical solution for a better fractionation and detection by reacting strongly basic proteins as amine
35   component with a low molecular weight carboxylic acid R_C-CO_2H, isonitrile R_I-NC and carbonyl compound $R_1-C(=O)-R_2$, at least one of the latter three components each containing, for example, dyes or biotin label. Analogously, strongly acidic proteins are reacted as carboxyl component with the amine R_A-NH_2, the isonitrile R_I-

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NC and the carbonyl compound R_1-C(=O)-R_2. Glycoproteins are reacted in oxidized form as carbonyl component with the amine R_A-NH_2, the carboxylic acid R_C-CO_2H and the isonitrile R_I-NC. Thus there are three novel possibilities of generating patterns of protein mixtures, it being possible to identify the newly formed protein groupings by the attached label. Consecutive application of all modifications would make it possible to simultaneously classify corresponding groups of proteins on the basis of their different labels.

The invention is explained in more detail by the following exemplary embodiments.

Example 1

Preparation of (Ia) for P = bovine serum albumin (BSA), R_1, R_2 = CH_3, R_I = cyclohexyl, R_A = 5-(β-D-glucopyranosyloxy)pentyl, n = 7.7.

A solution of bovine serum albumin (1 mg, 0.015 μmol) in water (1 ml), 5% strength acetone solution in water (67.2 μl, 45 μmol), 5-aminopentyl β-D-glucopyranoside (13.59 mg, 45 μmol) and 2 drops of cyclohexyl isonitrile are added to 3 ml 0.1 molar phosphate buffer, pH 7.5. The solution is left at room temperature for 2 days and concentrated in an ultrafiltration cell. The crude protein conjugate is subsequently dialyzed once against 0.1 molar phosphate buffer, pH 7.5, and twice against pure water. The molar mass of the product is determined by means of MALDI-TOF mass spectrometry: molecular weight: 69978 Da, i.e. a mean epitope density of n=7.7.
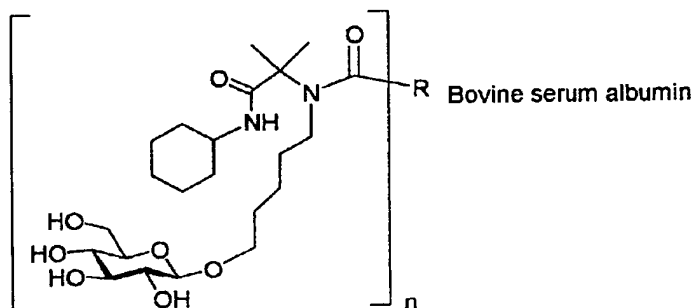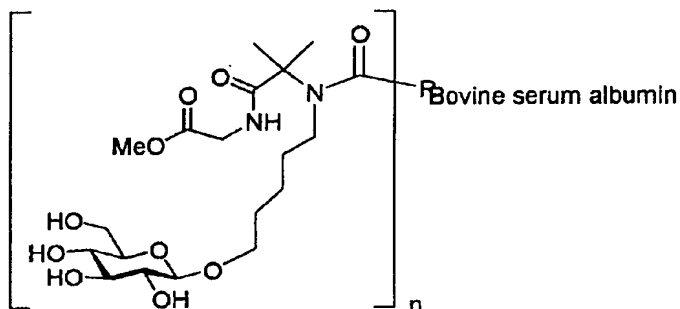

Example 2

Preparation of (Ia) for P = bovine serum albumin (BSA), R_1, R_2 = CH_3, R_I = cyclohexyl, R_A = 5-(β-D-glucopyranosyloxy)pentyl, n = 1.0.

Analogously to Example 1, bovine serum albumin (1 mg, 0.015 µmol), 5% strength acetone solution in water (2.24 µl, 1.5 µmol), 5-aminopentyl β–D-glucopyranoside (0.45 mg, 1.5 µmol) and 1 drop of cyclohexyl isonitrile are reacted in 0.1 molar Tris buffer, pH 7.5. Result: molecular weight: 66898 Da, mean epitope density: n=1.0; for formula see Example 1.

5

Example 3

Preparation of (Ia) for P = bovine serum albumin (BSA), R_1, R_2 = CH_3, R_I = methoxycarbonylmethyl, R_A = 5-(β-D-glucopyranosyloxy)pentyl, n = 8.8.

10 Analogously to Example 1, bovine serum albumin (1 mg, 0.015 µmol), 5% strength acetone solution in water (22.4 µl, 15 µmol), 5-aminopentyl β–D-glucopyranoside (4.5 mg, 15 µmol) and 1 drop of methyl isocyanoacetate are reacted in 0.1 molar phosphate buffer, pH 7.5. Result: molecular weight: 67948 Da, i.e. mean epitope density: n=3.4.

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Example 4

Preparation of (Ia) for P = bovine serum albumin (BSA), R_1, R_2 = CH_3, R_I =

20 cyclohexyl, R_A = 5-(2-acetamido-2-deoxy β-D-glucopyranosyloxy)pentyl, n = 8.8.

Analogously to Example 1, bovine serum albumin (1 mg, 0.015 µmol), 5% strength acetone solution in water (44.8 µl, 30 µmol), 5-aminopentyl 2-acetamido-2-deoxy-β-D-glucopyranoside (10.28 mg, 30 µmol) and 2 drops of cyclohexyl isonitrile are reacted in 0.1 molar phosphate buffer, pH 7.5. Result: molecular weight: 70480 Da,
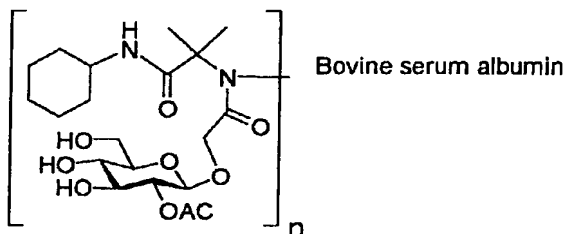
25 i.e. mean epitope density: n=8.8.

15
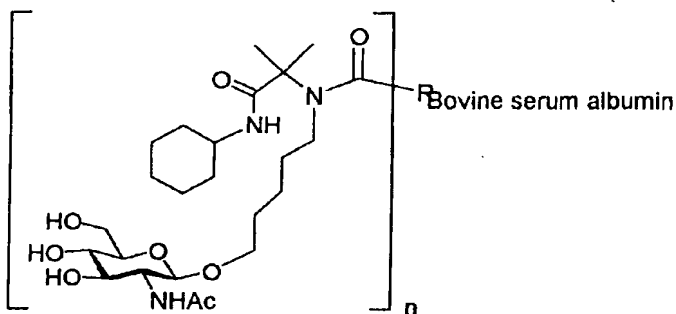
Example 5

Preparation of (Ib) for P = bovine serum albumin (BSA), R_1, R_2 = CH_3, R_I = cyclohexyl, R_C = (2-O-acetyl-β-D-glucopyranosyloxy)methyl, n = 9.9.

Analogously to Example 1, bovine serum albumin (1 mg, 0.015 μmol), 5% strength acetone solution in water (89.6 μl, 60 μmol), carboxymethyl 2-O-acetyl-β-D-glucopyranoside (19.1 mg, 60 μmol) and 2 drops of cyclohexyl isonitrile are reacted in 0.1 molar phosphate buffer, pH 7.5. Molecular weight: 70975 Da, mean epitope density: n=9.9.

Example 6

Preparation of (Ib) for P = bovine serum albumin (BSA), R_1, R_2 = CH_3, R_I = cyclohexyl, R_C = rhodamine B, n = 4.1-4.8.

Analogously to Example 1, bovine serum albumin (1 mg, 0.015 μmol), 5% strength acetone solution in water (67.2 μl, 45 μmol), rhodamine B (21.6 mg, 45 μmol, in 400 μl water) and 2 drops of cyclohexyl isonitrile are reacted in 0.1 molar phosphate buffer, pH 7.0, (3932.8 μl). Conjugate yield and mean epitope density are determined photometrically by measuring the extinctions at 280 nm (bovine serum albumin) and 575 nm (rhodamine B).

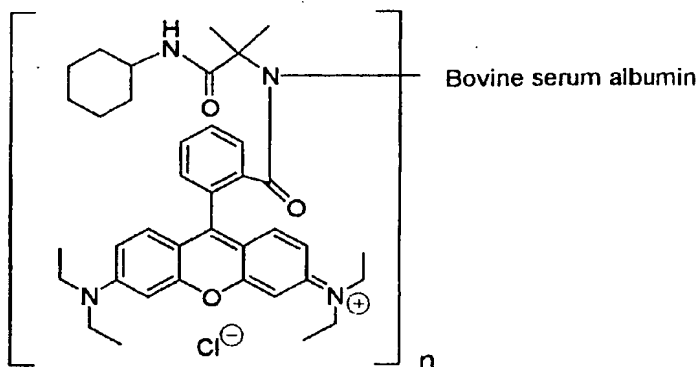Result after 1 day reaction time:

Bovine serum albumin:	7.4 nmol	Rhodamine B: 30.4 nmol
Conjugate yield: 49%		Mean epitope density: n=4.1

Result after 2 days reaction time:

Bovine serum albumin: 1.2 nmol   Rhodamine B: 6.0 nmol

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Conjugate yield: 8.3%   Mean epitope density: n=4.8

Bovine serum albumin

5   Example 7

Preparation of (Ib) for P = bovine serum albumin (BSA), R_1, R_2 = CH_3, R_1 = 1β-D-glucopyranosyl, R_C = rhodamine B, n = 6.8-12.5.

Analogously to Example 1, bovine serum albumin (1 mg, 0.015 μmol), 5% strength acetone solution in water (67.2 μl, 45 μmol), rhodamine B (400 μg, 0.835 μmol, in
10   400 μl water) and β-D-glucopyranosyl isonitrile (1.9 mg, 10 μmol, in 1 ml water) are reacted in 0.1 molar phosphate buffer, pH 7.0 (2932.8 μl). β-D-glucopyranosyl isonitrile is freshly prepared by dissolving 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisonitrile (3.6 mg, 10 μmol) in 5 ml methanol, 5 h stirring at RT with catalytic amounts of NaOMe, neutralizing by Dowex H+ ion exchanger, filtration and
15   concentration of the filtrate. Conjugate yield and mean epitope density are determined photometrically by measuring the extinctions at 280 nm (bovine serum albumin) and 575 nm (rhodamine B).

Result after 2 days reaction time:

Bovine serum albumin: 15 nmol   Rhodamine B: 6.8 nmol
20   Conjugate yield: 100%   Mean epitope density: n=0.45

Result after 4 days reaction time:

Bovine serum albumin: 12 nmol   Rhodamine B: 12.5 nmol

Conjugate yield: 80%   Mean epitope density: n=1.04

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Bovine serum albumin
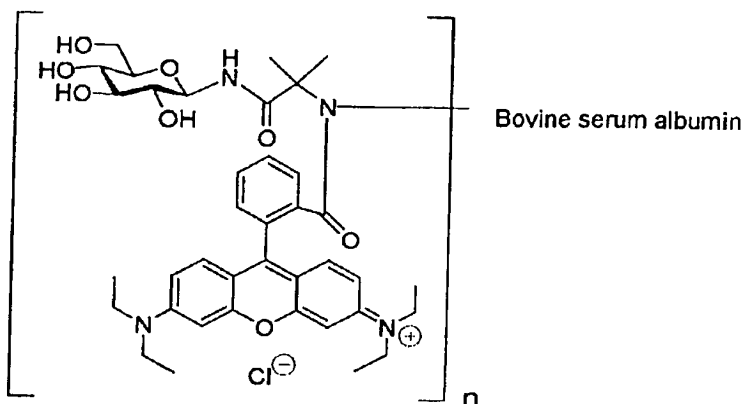
Example 8

Preparation of (Ib) for P = horseradish peroxidase (HRP), R_1 = H, R_2 = CH_2CH_3, R_I = cyclohexyl, R_C = 5-(biotinoylamino)pentyl, n = 3.0

Analogously to Example 1, horseradish peroxidase (1.9 mg, 0.049 µmol HRP), (+)-biotinaminocaproic acid (14.5 mg, 45 µmol), propionaldehyde (2.4 µg, 0.05 µmol) and cyclohexyl isonitrile (2 drops) are reacted in 0.1 molar phosphate buffer, pH 7.5 (4000 µl). Conjugate yield is determined photometrically by measuring the extinction at 280 nm. The residual activity of the peroxidase conjugate compared to native peroxidase is determined photometrically by oxidation of an ABTS dye solution and extinction measurement at 404 nm. The degree of biotinylation is determined quantitatively in a photometer by displacing HRP-BA from its avidin complex and measuring the extinction at 500 nm and qualitatively by an ELISA assay in streptavidin-coated microtiter plates.
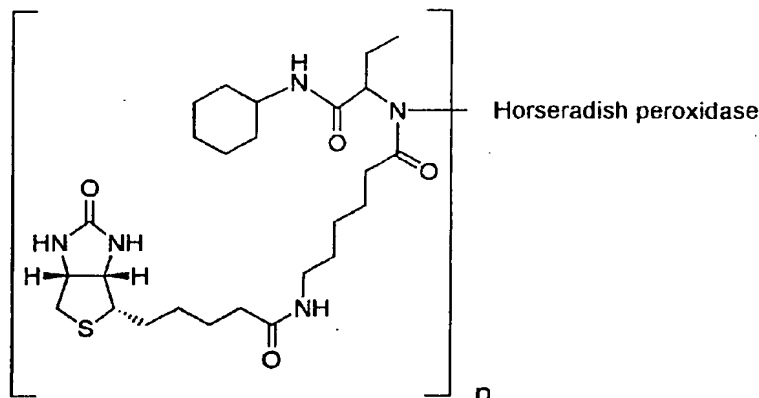
Result after 1 day reaction time:

Peroxidase conjugate: 36 nmol (75%)   Residual activity: 62%

Biotin content: 108 nmol   Epitope density: n=3.0

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Horseradish peroxidase

Example 9

Preparation of (Ic) for P = ox-horseradish peroxidase (ox-HRP), R_I = cyclohexyl,

R_C = rhodamine B, R_A = = 5-(biotinoylamino)pentyl, n = 3.3-3.4

Analogously to Example 1, NalO$_4$-oxidized horseradish peroxidase (1.8 mg, 0.045 µmol), (+)-biotinamidopentylamine (14.8 mg, 45 µmol), rhodamine B (21.6 mg, 45 µmol) and cyclohexyl isonitrile (2 drops) are reacted in 0.1 molar phosphate buffer, pH 7.5 (4000 µl). Conjugate yield is determined photometrically by measuring the extinction at 280 nm (peroxidase conjugate) and 575 nm (rhodamine B). The residual activity of the peroxidase conjugate compared to native peroxidase is determined photometrically by oxidation of an ABTS solution and extinction measurement at 404 nm. The degree of biotinylation is determined quantitatively in a photometer by displacing ox-HRP-BA from its avidin complex and measuring the extinction at 500 nm and qualitatively by an ELISA assay in streptavidin-coated microtiter plates.

Result after 1 day reaction time:

Molecular weight: 46118 Da   Epitope density: n = 3.4

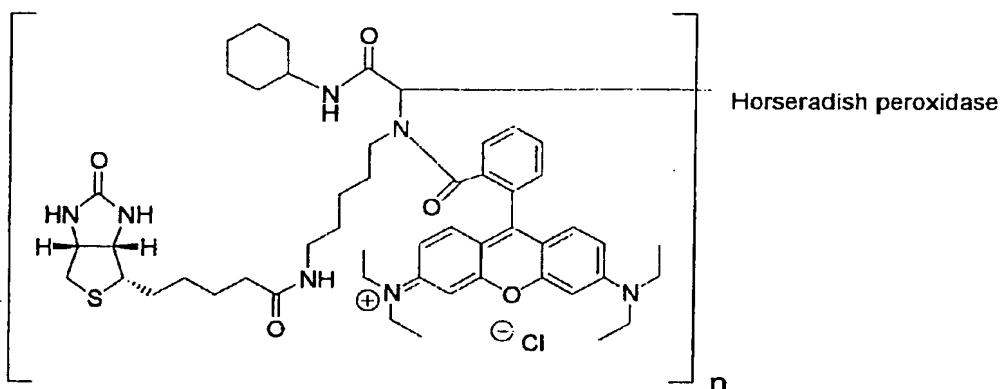Peroxidase conjugate: 4.8 nmol (11%)   Residual activity: 93%

Rhodamine B content: 16.8 nmol   Epitope density: n=3.5

Biotin content: 16 nmol   Epitope density: n=3.3

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Example 10

Preparation of (Ic) for P = ox-horseradish peroxidase (ox-HRP), R_I = cyclohexyl,

R_C = 5-(biotinoylamino)pentyl, R_A = = 5-(biotinoylamino)pentyl, n = 1.0

Analogously to Example 1, $NaIO_4$-oxidized horseradish peroxidase (1 mg, 0.026 µmol), (+)-biotinamidopentylamine (14.8 mg, 45 µmol), (+)-biotinaminocaproic acid (14.5 mg, 45 µmol) and cyclohexyl isonitrile (2 drops) are reacted in 0.1 molar phosphate buffer, pH 7.5 (4000 µl). Conjugate yield is determined photometrically by measuring the extinction at 280 nm. The residual activity of the peroxidase conjugate compared to native peroxidase is measured photometrically by oxidation of an ABTS solution and extinction measurement at 404 nm. The degree of biotinylation is determined quantitatively in a photometer by displacing ox-HRP-BA from its avidin complex and measuring the extinction at 500 nm and qualitatively by an ELISA assay in streptavidin-coated microtiter plates.
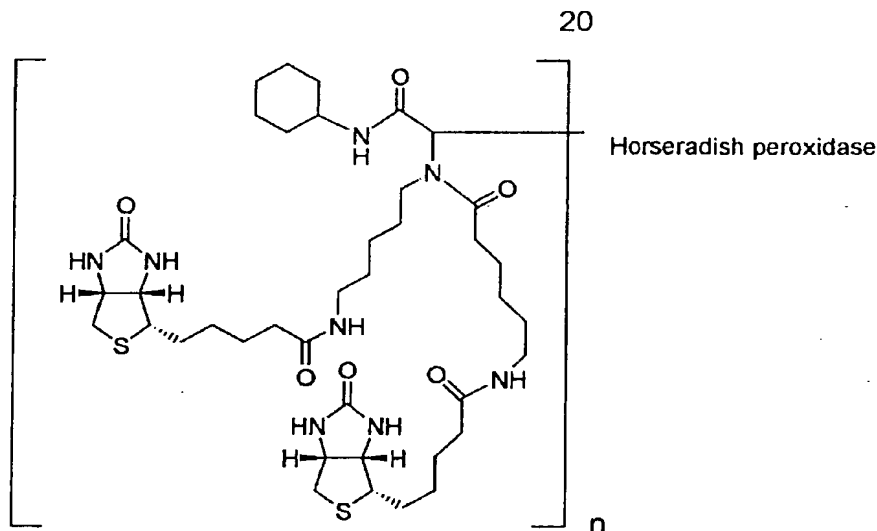
Result after 1 day reaction time:

Peroxidase conjugate: 16 nmol (67%)   Residual activity: 157%

Biotin content: 16 nmol   Epitope density: n=1.0

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Horseradish peroxidase

Example 11

Proteome analysis: The soluble proteins of a microorganism, for example from yeast
or *E.coli* cells, or a mammalian organism, for example from human blood serum, are
obtained according to the familiar methods (cell lysis, extraction, washing in buffers,
etc.). Instead of being directly applied to an electrophoresis gel the protein mixture is
derivatized beforehand in aqueous buffer, quite analogously to the description in one
of the Examples above. After two-dimensional fractionation the gel is developed as
usual, for example by Coomassie blue, silver nitrate or a fluorescent dye. If at least
one of the three employed low molecular weight reaction components is already a
derivative of a suitable fluorescent dye, then the corresponding modified proteins
may be detected directly in a reader. Image processing (e.g. by means of the Biorad
program Melanie II, PC-NT) and the subsequent computer-based processing (i.e.
pattern recognition and analysis) are carried out as is usual for the analysis of two-
dimensional electrophoresis chromatograms. Additionally, it is possible to pick up the
gel spots of interest as usual by a gel picker and to analyze them in a mass
spectrometer after trypsin digestion (MALDI-TOF, ESI, MS-MS).

Patent Claims:

1.   A protein conjugate of the structure given in formulae (Ia-c),
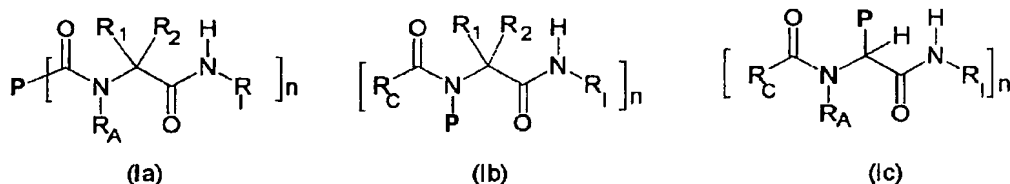
(Ia)   (Ib)   (Ic)

wherein **P** is a protein carrying free amino, carboxyl or aldehyde groups such as an albumin, immunoglobulin, antibody, avidin, streptavidin, hemocyanin, lectin, enzyme or serum glycoprotein,

and R_A is a radical derived from amines R_A-NH_2, where R_A is an amino group-carrying linear or branched alkyl or cycloalkyl radical of up to 18 carbon atoms, an amino group-carrying alkaloid, peptide or protein, carbohydrate, nucleotide, nucleoside, steroid, terpene, porphyrin, chlorin, corrin, eicosanoid, pheromone, vitamin, in particular an n-(biotinamido)-n-alkyl radical where n = 2-8, an antibiotic, cytostatic, a dye molecule or a cryptand,

and R_C is a radical derived from carboxylic acids R_C-CO_2H, where R_C is a carboxyl group-carrying linear or branched alkyl or cycloalkyl radical of up to 18 carbon atoms, a carboxyl group-carrying alkaloid, peptide or protein, carbohydrate, nucleotide, nucleoside, steroid, terpene, porphyrin, chlorin, corrin, eicosanoid, pheromone, vitamin, in particular biotin, an antibiotic, cytostatic, a dye molecule or a cryptand,

and R_I is a radical derived from isonitriles R_I-NC, where R_I is an isonitrile group-carrying linear or branched alkyl or cycloalkyl radical of up to 18 carbon atoms, methoxycarbonylethyl, t-butoxycarbonylmethyl, phenyl, o-alkylphenyl, m-alkylphenyl, p-alkylphenyl, o-halophenyl, m-halophenyl, p-halophenyl, 2,3-dihalophenyl, 2,4-dihalophenyl, 3,4-dihalophenyl, o-alkoxyphenyl, m-alkoxyphenyl, p-alkoxyphenyl, o-arylphenyl, m-arylphenyl, p-arylphenyl, o-aryloxyphenyl, m-aryloxyphenyl, p-aryloxyphenyl, o-nitrophenyl, m-nitrophenyl, p-nitrophenyl, 1-naphthyl, 2-naphthyl, benzenesulfonylmethyl, p-toluenesulfonylmethyl, pyranosyl radical, furanosyl radical, nucleosyl radical, n-(biotinamido)-n-alkyl radical where n = 2-8 or a dye molecule,

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and R_1 and R_2 are a radical R_1-C(=O)-R_2 derived from carbonyl compounds and can be simultaneously or independently of one another hydrogen, methyl, ethyl, propyl, i-propyl, cyclopropyl, butyl, i-butyl, t-butyl, cyclobutyl, pentyl, cyclopentyl, hexyl, cyclohexyl, 2-methylbutyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl,

5   methoxycarbonylethyl, t-butoxycarbonylmethyl, phenyl, o-alkylphenyl, m-alkylphenyl, p-alkylphenyl, o-halophenyl, m-halophenyl, p-halophenyl, 2,3-dihalophenyl, 2,4-dihalophenyl, 3,4-dihalophenyl, o-alkoxyphenyl, m-alkoxyphenyl, p-alkoxyphenyl, o-arylphenyl, m-arylphenyl, p-arylphenyl, o-aryloxyphenyl, m-aryloxyphenyl, p-aryloxyphenyl, o-nitrophenyl, m-nitrophenyl, p-nitrophenyl, 1-naphthyl, 2-naphthyl,

10   oxiranyl, vinyl, propenyl, propen-2-yl, 2-penten-2-yl, 3-hepten-3-yl, penta-1,3-dienyl, phenylmethyl, 1-phenylethyl, 2-phenylethyl, cyclohexen-3-yl, cyclohexen-4-yl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyrrolyl, 3-pyrrolyl, halomethyl, dihalomethyl, trihalomethyl, 2-pyridyl, 3-pyridyl, 4-pyridyl

15   and n = 1-15.

2.   A process for preparing the protein conjugates as claimed in claim 1 which comprises reacting a protein (P) which is soluble or immobilized on a solid phase and which carries free amino, carboxylic acid or aldehyde groups with an amine R_A-

20   NH_2, or carboxylic acid R_C-CO_2H, isonitrile R_I-NC and carbonyl compound R_1-C(=O)-R_2, where R_A, R_C, R_I, R_1 and R_2 are as stated in claim 1, in aqueous solution to give the relevant protein conjugates (Ia), (Ib) or (Ic), the respective products (Ia) being obtained from a protein (P), amine R_A-NH_2, isonitrile R_I-NC and carbonyl compound R_1-C(=O)-R_2, the products (Ib) from the protein (P), a carboxylic acid

25   R_C-CO_2H, isonitrile R_I-NC and carbonyl compound R_1-C(=O)-R_2, and the products (Ic) from an oxidized protein (P), a carboxylic acid R_C-CO_2H, amine R_A-NH_2 and isonitrile R_I-NC, where R_A, R_C, R_I, R_1 and R_2 are as stated in claim 1.

3.   The process as claimed in claim 2, wherein the protein (P) is reacted with the

30   amine R_A-NH_2 or the carboxylic acid R_C-CO_2H, and the isonitrile R_I-NC and the carbonyl compound R_1-C(=O)-R_2 by reacting the protein (P) dissolved or suspended in aqueous buffer with the amine R_A-NH_2 or the carboxylic acid R_C-CO_2H and the isonitrile R_I-NC and the carbonyl compound R_1-C(=O)-R_2, each being employed in a 10 - 10,000 fold molar excess over the protein (P).

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4.   The process as claimed in claims 2-3, wherein the reaction takes place in aqueous buffer solutions, in particular in 0.001 - 1.0 molar solutions of sodium or potassium dihydrogen phosphate and disodium or dipotassium hydrogen phosphate

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or in buffer solutions of tris(hydroxymethyl)aminomethane and hydrochloric acid, in a pH range of between 5 and 9.

5.   The process as claimed in claims 2-4 wherein the reaction buffer further contains cosolvents such as methanol, ethanol, propanol, i-propanol, butanol, ethyl acetate, methyl acetate, dimethyl formamide, acetonitrile, dimethyl sulfoxide or sulfolane in quantities of 0.1-20% by volume and the reaction temperatures are between 0°C and 90°C.

6.   The use of the protein conjugates (Ia-c) as claimed in claim 1 in immunoassays, synthetic vaccines or as biosensors.

7.   The therapeutic use of the protein conjugates (Ia-c) as claimed in claim 1 in which at least one of the radicals R_C, R_A, R_I, R_1 or R_2 is a cytostatic bound to a tumor-specific antibody or is an enzyme or lectin.

8.   The use of the process as claimed in claims 2 to 5 for modifying protein mixtures in the preparation of samples for modified proteome analysis of organisms by means of electrophoresis.

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